Update on U.S. progress on *in vitro* thyroid-axis endpoint screening assays

17th Meeting of the Validation Management Group on Non-animal Testing

OECD, Advisory Group on Endocrine Disrupters Testing and Assessment (EDTA) of the Test Guidelines Programme.

Virtual Meeting/Teleconference

Thursday, November 7, 2019

The views expressed in this presentation are those of the author and do not necessarily reflect the views or policies of the US EPA.
Thyroperoxidase Inhibition
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Radioactive Iodide Uptake Inhibition
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Jun Wang (ORISE)²
Dan Hallinger¹
Ashley Murr²
Angela Buckalew²

TSHR / TRHR
Katie Paul-Friedman

Thyronine and Tyrosine Deiodinase Inhibition
Michael Hornung¹ (presenting)
Sigmund Degitz¹
Jennifer Olker¹
Sally Mayasich (ORISE)¹
Jeff Denny¹
Joe Korte¹
Phillip Hartig²
Mary Cardon²

Thyroid Hormone Activation
Katie Paul-Friedman¹
Keith Houck¹
Thyroid Axis

- **Hypothalamus**
- **Pituitary**
- **Thyroid**
- **Blood**
- **Liver**

**Control**

**Synthesis**

**Hormone Transport**

**Metabolism and Elimination**

**Peripheral Tissues**
- Deiodinases – Activation/Inactivation
- Receptor Activation

→ TH Action
Screening Assays

Control
- TSHR → stimulation of hormone production
- TRHR

Synthesis
- TPO Inhibition → hormone iodination and coupling
- NIS Inhibition → iodide uptake
- IYD Inhibition → iodide recycling

Signaling
- DIO 1, DIO2, DIO3 Inhibition → hormone activation / inactivation control
- Thyroid hormone receptor transactivation assays → ultimate signaling

HTP screening assays in Tox21, but more work needed to establish specificity.
Thyroid Hormone Synthesis

Synthesis MIEs

**Iodide Transport**
- Sodium-Iodide Symporter (NIS)
- Pendrin (PEN)

**Tyrosyl Iodination and Coupling**
- Thyroperoxidase (TPO)
- Dual Oxidase (DUOX)

**Iodide Recycling**
- Iodotyrosine Deiodinase (IYD)
Thyroperoxidase Inhibition

- TPO in the thyroid gland catalyzes tyrosine iodination & coupling in synthesis of T4 & T3.
- Evaluated commercially-available peroxidase substrates to find a substitute for guaiacol that was HTS-amenable:
  - **Amplex UltraRed (AUR)** met criteria needed for a HTS substrate
- Rat thyroid gland microsomes were source of TPO for this screening
- Recombinant TPO source is promising
Single-concentration screen for TPO inhibition activity:

1,900 ToxCast chemicals

RESULT: 26% of screened chemicals produced greater than 20% inhibition

... or 74% of chemicals produced less than 20% inhibition and likely inactive

- TC Phase 1 (300 chemicals)
- TC Phase 2 (800 chemicals)
- TC E1K (800 chemicals)
Thyroid Hormone Synthesis - NIS

**Iodide Uptake Inhibition**

NIS = Sodium/iodide (Na⁺/I⁻) symporter

- Mediates thyroid gland iodide uptake
- Known target of environmental contaminants (ex. Perchlorate, ClO₄⁻)
- Limited knowledge for more structurally diverse chemicals

**Radioactive Iodide Uptake Assay (RAIU)**

- Cell Culture: hNIS-HEK293T-EPA
- Radioactive ¹²⁵Iodide Uptake X Cell Viability
- Measure ¹²⁵I uptake X Cell Viability by ATP

Excess Perchlorate can inhibit NIS-mediated iodide uptake by diminishing the [Na⁺], causing low intracellular [Na⁺] maintained by Na⁺/K⁺-ATPase.
Radioactive Iodide Uptake Inhibition

ToxCast phase 1, 2, and e1K libraries

**Single-Concentration Screening**

- **1896 Samples**
  - RAIU assay (100 µM)

**Selection**

- > 20% inhibition

**Multi-Concentration Screening**

- **618 Samples**
  - RAIU & Cytotox Assays
    - (0.001 - 100 µM)

**Median and Range of Test Chemical Responses**

(Single Concentration Screening)

Chemicals, ordered by increasing iodide uptake inhibition

618 (32.6% out of 1896) Samples Selected for Multi-Concentration RAIU and Cell Viability Assays
Quantitative Chemical Ranking for NIS Inhibitors

Example Curves and Ranking Scores
NIS Screening – Multi-concentration results

Ranking Scores

Conc.-resp. curves for example chemicals with high ranking scores
IYD Inhibition Assay

Recombinant human IYD (hIYD) Enzyme Production

Produced with baculovirus system in insect cells

Xenopus IYD (xIYD) Enzyme Source

Liver microsomal fractions from *Xenopus laevis* tadpoles

IYD Enzyme Assay

In 96-well plates, 3 h incubation

Measure Detection Signal

Sandell-Kolthoff (SK) reaction to detect free iodide

Iodotyrosine deiodinase (IYD) reaction scheme

3-Nitro-L-tyrosine (MNT) as positive control

Liver microsomal fractions from *Xenopus laevis* tadpoles

As³⁺ + 2Ce⁴⁺ yellow → As⁵⁺ + 2Ce³⁺ colorless
IYD Inhibition Assay Screening

ToxCast ph1_v2, ph2, and e1K chemicals across all plates

≥ 20% inhibition
195 unique chemicals (10.7% of 1,828 tested)

% Inhibition for highest tested concentration of each chemical, clustered hierarchically

Human IYD Screening

% of Control

20% inhibition
50% inhibition

Chemicals, ordered by % inhibition (low to high)
Deiodinase Inhibition Assay

- Use adenovirus expression system in HEK293 cells to obtain active DIO1, 2, and 3 enzymes.
- Deiodinase assay incubated in 96-well plate followed by 96-well solid phase Dowex columns to extract and isolate iodide.
- Sandell-Kolthoff reaction used to quantify iodide (same as for IYD assay).
- Inhibition of the DIO reduces amount of iodide, resulting in reduced rate of absorbance change.
Hormone Activation / Inactivation

- Deiodinase Inhibition Assay

<table>
<thead>
<tr>
<th>Chemical Library</th>
<th># chemicals tested</th>
<th># with ≥ 20% inhibition</th>
<th>% with ≥ 20% inhibition</th>
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<th>% with ≥ 20% inhibition</th>
<th># with ≥ 20% inhibition</th>
<th>% with ≥ 20% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ToxCast p1_v2</td>
<td>290</td>
<td>49</td>
<td>16.9 %</td>
<td>54</td>
<td>18.6 %</td>
<td>57</td>
<td>19.7 %</td>
</tr>
<tr>
<td>ToxCast p2</td>
<td>748</td>
<td>95</td>
<td>12.7 %</td>
<td>126</td>
<td>16.8 %</td>
<td>117</td>
<td>15.6 %</td>
</tr>
<tr>
<td>ToxCast e1K</td>
<td>781</td>
<td>77</td>
<td>9.9 %</td>
<td>123</td>
<td>15.8 %</td>
<td>133</td>
<td>17.1 %</td>
</tr>
<tr>
<td>Total</td>
<td>1,819</td>
<td>221</td>
<td>12.1 %</td>
<td>303</td>
<td>16.7 %</td>
<td>307</td>
<td>16.9 %</td>
</tr>
</tbody>
</table>
hDIO Inhibition

1819 chemicals: hierarchical clustering by % inhibition at single concentration

DIO1 DIO2 DIO3

DIO1 DIO2 DIO3

DIO1 DIO2 DIO3

% of Control (DMSO)

Concentration, µM
Thyroid Hormone Receptor Transactivation

- Primary screen in GH3-TRE-Luc cell line (A. Murk)
- 8300 unique structures screened in Tox21 library
- Assays run in agonist and antagonist mode
- Confirmatory and orthogonal assays run to verify actives
  - TR:Coactivator recruitment assays
  - GAL4-LBD (human) reporter gene assay
  - RXR reporter gene assay
  - TR-GR-GFP nuclear translocation assay (G. Hagar lab)
  - Cytotoxicity assays
Receptor Screening Results

Agonists
- Direct agonists—T3 analogs
- Indirect agonists—RXR ligands

Antagonists
- Only 3 high confidence antagonists, all pharmaceutical class compounds
- Suspected indirect antagonists such as proteasome inhibitors also identified
- Nuclear translocation assay very useful as orthogonal assay
<table>
<thead>
<tr>
<th>MIE</th>
<th># Chemicals Tested</th>
<th>Data Status</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIS Radioactive Iodide Uptake Inhibition</td>
<td>&gt;1,800 (ToxCast ph1v2, ph2, e1k)</td>
<td>multi-concentration data for ph1v2 and ph2 available online</td>
<td>Hallinger et al. 2017 Wang et al. 2018 Wang et al. 2019</td>
</tr>
<tr>
<td>Thyroperoxidase Inhibition</td>
<td>&gt;1,800 (ToxCast ph1v2, ph2, e1k)</td>
<td>multi-concentration data available online</td>
<td>Paul-Friedman et al. 2016</td>
</tr>
<tr>
<td>Iodothyronine Deiodinase Type 1</td>
<td>&gt;1,800 (ToxCast ph1v2, ph2, e1k)</td>
<td>multi-concentration data available online in next update of InVitroDB</td>
<td>Hornung et al. 2018 Olker et al. 2019</td>
</tr>
<tr>
<td>Iodothyronine Deiodinase Type 2</td>
<td>&gt;1,800 (ToxCast ph1v2, ph2, e1k)</td>
<td>multi-concentration data available online in future update of InVitroDB</td>
<td>Olker et al. 2019</td>
</tr>
<tr>
<td>Iodothyronine Deiodinase Type 3</td>
<td>&gt;1,800 (ToxCast ph1v2, ph2, e1k)</td>
<td>multi-concentration data available online in future update of InVitroDB</td>
<td>Olker et al. 2019</td>
</tr>
<tr>
<td>Iodotyrosine Deiodinase</td>
<td>&gt;1,800 (ToxCast ph1v2, ph2, e1k)</td>
<td>Analysis in progress, to be submitted to InVitroDB early 2020</td>
<td>Olker et al. In prep.</td>
</tr>
<tr>
<td>Thyroid Hormone Receptor Transactivation</td>
<td>8,300</td>
<td>Source data available on InVitroDB</td>
<td>Paul-Friedman et al. 2019</td>
</tr>
</tbody>
</table>
Nearly two dozen molecular initiating events (MIE) have been identified/proposed for thyroid axis adverse outcome pathways; about half have high-throughput screening assay available or being developed for the MIE.

Future efforts needed to translate *in vitro* activity to *in vivo* responses to verify Adverse Outcomes.

Incorporate *in vitro* screening date for MIE and AOP into framework for use in risk assessment for chemical disruption of thyroid hormones

- prioritization near-term goal
- ultimately inform & develop quantitative AOP to predict toxicity

**Recent commentary on this effort.**
https://doi.org/10.1289/EHP5297
Publicly available data

iCSS ToxCast dashboard

https://comptox.epa.gov/dashboard

Invitrodb database

https://doi.org/10.23645/epacomptox.6062623.v1

Data related to published papers also available on www.data.gov
Publications

**TPO**

**NIS**

**DIO**
- Hornung et al. 2018. Screening the ToxCast Phase 1 Chemical Library for Inhibition of Deiodinase Type 1 Activity. *Toxicological Sciences.* 162(2), 570-581.
- Olker et al. 2019 Screening the ToxCast Phase 1, Phase 2, and e1k Chemical Libraries for Inhibitors of Iodothyronine Deiodinases. *Toxicol Sci* 168(2):430-442.

**IYD**

**THR Transactivation**

**Review / Commentary**